IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re application of:

MICHEL GILBERT et al.

Application No.: 09/211,691

Filed: December 14, 1998

For: FUSION PROTEINS FOR USE IN ENZYMATIC SYNTHESIS OF OLIGOSACCHARIDES

Customer No.: 20350

Confirmation No. 9572

Examiner: Rao, Manjunath

Technology Center/Art Unit: 1652

DECLARATION OF DR. MICHEL GILBERT UNDER 37 C.F.R. §1.132

Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

Sir:

I, Michel Gilbert, Ph.D., being duly warned that willful false statements and the like are punishable by fine or imprisonment or both (18 U.S.C. § 1001), and may jeopardize the validity of the patent application or any patent issuing thereon, state and declare as follows:

1. All statements herein made of my own knowledge are true, and statements made on information or belief are believed to be true and correct.

2. I, Dr. Gilbert, am currently Research officer at the Institute for Biological Sciences in the Immunochemistry Program of the Pathogen Genomics Group at the National Research Council of Canada (NRC). I received my B.Sc. in Microbiology from the Université Laval, Département de biochimie et microbiologie in Québec, Canada in 1986. I received my Ph.D. in Ph.D. in Biology (biochemistry specialization) from the University of Ottawa, Department of Biology in 1992. I was a post doctoral fellow in the Laboratory of Dr. Rolf Morosoli at the Institut Armand-Frappier of Laval, Québec from 1993-1994. I joined the NRC in 1995 as a research associate in the laboratory of Dr. Warren Wakarchuk at the NRC laboratories in Ottawa as part of the Immunochemistry Program. In 1999, I was appointed

APPENDIX A

Associate Research Officer of the NRC, the position I hold today. A copy of my curriculum vitae is attached hereto as Exhibit B.

3. The present invention is nucleic acids that encode fusion proteins comprising an α-2,3-sialyltransferase that catalyzes the transfer of a sialic acid, from CMP-Neu5Ac, to an acceptor molecule; and a CMP-Neu5Ac synthetase that catalyzes the formation of CMP-Neu5Ac from Neu5Ac and CTP.

4. I am a named inventor on the above-referenced patent application. I have read and am familiar with the contents of this patent application. In addition, I have read an Office Action, dated May 26, 2004 and a related Advisory Action, dated November 22, 2004, received in the present case. It is my understanding that the Examiner alleges that the claimed invention is obvious in view of Bulow *et al.*, *TIBtech* 9:226-231 (1991); Defrees *et al.*, WO 96/32491; and the common knowledge of the art of molecular biology provided by Sambrook *et al.*, pages 7.37-7.52 (1989) in further view of Gilbert(a) *et al. Eur. J. Biochem.*, 249:187-194 (1997) and Gilbert(b) *et al. Biotech. Lett.* 19:417-420 (1997). In particular, the Examiner appears to believe that certain advantages of the claimed invention are not unexpected given the teachings of Bulow *et al.* and he also alleges that the practical and statistical significance of those advantages has not been established.

5. This declaration is provided to demonstrate that the advantages of the claimed invention, *e.g.*, increased activity and solubility of fusion protein components compared to unfused proteins, are unexpected in view of the teachings of Bulow *et al.* and the other cited references and that the advantages described in the specification and herein are of practical and statistical significance.

6. Bulow *et al.* fail to demonstrate that an increase in activity of individual fusion protein components can be expected on expression of a fusion protein. Bulow *et al.* discuss improvements in a coupled reaction activities, relying on *e.g.*, proximity effects for the difference between unfused enzymes and fused enzymes. In addition, Bulow *et al.* at page 230, first full paragraph states that "When corrected for the increase in molecular weight caused by

the fusion, the specific activities correspond to 50-100% of those of native enzymes." Thus, Bulow *et al.* expect no increase in the specific activity of a fused protein and, apparently hope that the presence of a fusion partner will not interfere with the activity observed for the unfused enzymes. Bulow *et al.* teach that even 50% of the unfused protein activity would be acceptable, provided that other characteristics of the fused proteins change only modestly, *e.g.*, Km, pH-optima, thermostability, and stability to urea denaturation. *Id.* In addition, Bulow *et al.* do not disclose improvements in solubility of an insoluble protein when expressed as part of a fusion protein.

7. We observed an unexpected and significant increase in the specific activity of both the α-2,3-sialyltransferase and the CMP-NeuAc synthetase after they were expressed as a fusion protein. *See, e.g.*, specification at page 46, lines 1-8. These increases were measured in uncoupled reactions. The fused and unfused α-2,3-sialyltransferase have different molecular weights. In order to correct for the increased molecular weight of the fusion protein, we expressed the activity as the turnover rate of the enzyme. The turnover rate of the unfused α-2,3-sialyltransferase is 1.4 s^{-1}; the turnover rate of the fused α-2,3-sialyltransferase is 3.2 s^{-1}. Thus, the activity of the fused α-2,3-sialyltransferase corresponds to 229% of the activity of the unfused enzyme. The specific activity of the CMP-NeuAc synthetase also increased after fusion to the α-2,3-sialyltransferase. The turnover rate of the unfused CMP-NeuAc synthetase is 31.4 s^{-1}; the turnover rate of the fused CMP-NeuAc synthetase is 39.5 s^{-1}. Thus, the activity of the fused CMP-NeuAc synthetase corresponds to 126% of the activity of the unfused enzyme. The unexpected increases in α-2,3-sialyltransferase and the CMP-NeuAc synthetase after fusion are both practically and statistically significant.

8. We also observed an unexpectedly large increase in the solubility of the fused α-2,3-sialyltransferase as compared to the unfused protein. *See, e.g.*, specification at page 45, lines 14-19. Unfused α-2,3-sialyltransferase precipitated out of solution at concentrations above 1 mg/ml, even in the presence of detergent. In contrast, α-2,3-sialyltransferase fused to CMP-NeuAc synthetase was soluble to 5 mg/ml in the presence of Triton X-100. We had hoped that fusion of the soluble CMP-NeuAc synthetase to the insoluble α-2,3-sialyltransferase would result in improved solubility of the fused α-2,3-sialyltransferase. However, we did not expect the

solubility of the α-2,3-sialyltransferase to increased five fold Moreover, the increased solubility of the α-2,3-sialyltransferase as part of a fusion protein has the practical effect of increasing the efficiency of oligosaccharide production. The low solubility of the unfused α-2,3-sialyltransferase makes it difficult to achieve the enzyme concentration required for efficient oligosaccharide synthesis, particularly on a large scale. In contrast, the fused protein can be used for efficient synthesis of oligosaccharides on a large scale. And, in fact, when the α-2,3-sialyltransferase/CMP-NeuAc synthetase fusion protein was used in large scale synthesis of sialyllactose, the reaction went to completion. See, *e.g.*, specification at page 48, lines 4-9. Therefore, the increase in solubility of α-2,3-sialyltransferase after fusion to CMP-NeuAc synthetase is both practically and statistically significant.

9. In my opinion the improvements in both enzymatic activities on fusion of α-2,3-sialyltransferase and CMP-NeuAc synthetase are unexpected in view of the disclosure of Bulow *et al.* and the other cited references. Bulow *et al.* teaches that no increase in fusion protein activity over unfused components was expected, when the molecular weights are considered, *i.e.*, when turnover numbers are compared. The results in the specification demonstrate that both α-2,3-sialyltransferase and the CMP-NeuAc synthetase proteins have increased turnover numbers as compared to unfused proteins. Bulow *et al.* do not teach improvements in solubility of an insoluble protein after fusion. The results in the specification also demonstrate that the fused α-2,3-sialyltransferase protein has five fold greater solubility than an unfused α-2,3-sialyltransferase protein. This large increase in solubility of the α-2,3-sialyltransferase was not expected. Thus, the improvements in activity and solubility of the α-2,3-sialyltransferase and the CMP-NeuAc synthetase proteins after fusion are unexpected in view of the cited references.

Date: January 29, 2005 By: Michel Gilbert

Michel Gilbert, Ph.D.

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Research Interests:

I am interested in studying glycoconjugate biosynthesis in microbial systems. The fundamental aspects include identifying the enzymes and the mechanisms that are involved in the synthesis of lipopolysaccharides, polysaccharides and glycoproteins. This knowledge will help to understand better the roles of glycoconjugates in pathogenesis and can also result in the development of carbohydrate-based therapeutic agents and vaccines.

Education:

1993-1994 Postdoctoral position: Institut Armand-Frappier (Laval, Québec) Programme de microbiologie appliquée, Supervisor : Dr. Rolf Morosoli. Topic : Study of the secretion system of *Streptomyces lividans*.

1986-1992 Ph.D. in Biology (biochemistry specialization): University of Ottawa, Department of Biology, Supervisor: Dr. J.N. Saddler. Title of the thesis: "Production and characterization of the cellulases and xylanases from the thermophilic ascomycete *Thielavia terrestris* 255B".

1983-1986 B.Sc. in Microbiology: Université Laval, Département de biochimie et microbiologie, Québec.

Experience:

1999-2005: Research officer : Institute for Biological Sciences, Immunochemistry Program, Pathogen Genomics Group. National Research Council of Canada.

-Supervisory experience: full-time supervision of a technical officer, a postdoctoral fellowship and of many summer and coop students. Co-supervision of a research associate and of many technical officers.

-Contributed to the writing of the research proposal for the "Genomics of Human Mucosal Pathogens" initiative.

-Directed the work on the comparative genomics studies of the lipooligosaccharide biosynthesis locus from *Campylopbacter jejuni*.

APPENDIX B

-Supervised the sequencing of various carbohydrate biosynthesis loci in *Campylobacter jejuni* and used various bio-informatics tools to analyse the sequences.

-Directed a study on the association between specific *Campylobacter jejuni* glycosyl-transferases and the development of Guillain-Barré and Miller-Fisher syndromes (in collaboration with Dr. Hubert Endtz, Erasmus University Medical Centre, Rotterdam, The Netherlands).

-Structure-function studies of various bacterial glycosyltransferases.

-Contributed to the development of chemi-enzymatic syntheses of carbohydrates under investigation in the carbohydrate-based vaccine program as part of a collaboration with Drs. Dennis Whitfield and Wei Zou.

-Collaboration with an industrial partner (Neose Technologies, Horsham, Pennsylvania): topic: development of glycosyltransferases for the synthesis of gangliosides and other glycoconjugates. Proposal writing, supervision of research activities, report writing and visits to their research facilities to present results and plan future work.

-Contributed to the writing of 5 patent applications (see below) in keeping with NRC strategy to insure proper protection of discoveries and technology developments resulting from research activities (intellectual property issues).

1995-1998: Research associate: Institute for Biological Sciences, Immunochemistry Program, Novel Antibodies and Proteins, National Research Council of Canada. Supervisor: Dr. W.W. Wakarchuk.

-Cloning of genes encoding bacterial glycosyltransferases that can be used for the synthesis of oligosaccharides with potential use as therapeutics. The cloning was performed using activity screening and genome analysis.

-Optimization of enzyme production by genetic engineering and optimization of fermentation conditions.

-Enzyme purification using chromatography techniques, ultrafiltration and precipitation techniques.

-Characterization of the acceptor specificity of the recombinant enzymes.

-Enzymatic synthesis of oligosaccharides with potential for use as therapeutics.

-Liquid and solid cultures of bacterial pathogens (BSL 2) such as *Neisseria meningitidis*, *Campylobacter jejuni* and pathogenic *Escherichia coli*.

-Collaboration with an industrial partner (Cytel Corporation, Glycotechnology division, San Diego, California): development of glycosyltransferases for the synthesis of oligosaccharides

with biological activities. I performed experiments, supervised research activities, wrote reports and presented our results at their research facilities. Development of a fusion enzyme that was used to synthesize kg quantities of siallylactose.

1993-1994: Postdoctoral position: Institut Armand-Frappier, Département de microbiologie appliquée. Superviseur : Dr. Rolf Morosoli. Topic : Study of the secretion system of *Streptomyces lividans*:

-Cloning, sequencing and characterization of genes encoding secretion factors.

-Production of recombinant proteins by prokaryotes (*Escherichia coli* and *Streptomyces lividans*).

-Site-directed mutagenesis.

1986-1992: Ph.D. thesis research (University of Ottawa, Department of Biology): the research was carried out at the Eastern Laboratory of Forintek Canada Corp.:

-Study of fungal enzymes (carbohydrate-degrading enzymes such as xylanases and cellulases) with potential industrial applications.

-Production and purification of enzymes.

-Enzyme characterization: physico-chemical properties and kinetics.

-Study of enzyme mechanisms and enzyme-enzyme interactions.

Professional Associations:

Member of the "Canadian Society of Microbiologists"

Patents:

Gilbert, M. and Wakarchuk, W.W. Polypeptides having β-1,3-Galactosyl transferase activity. U.S. Patent No. 6,825,019 B2 (Issued on November 30, 2004).

Gilbert, M. and Wakarchuk, W.W. Polypeptides having β-1,4-GalNAc transferase activity. U.S. Patent No. 6,723,545 (Issued on April 20, 2004).

Gilbert, M. and Wakarchuk, W.W. Lipopolysaccharide α-2,3-sialyltransferase of *Campylobacter jejuni* and its uses. U.S. Patent No. 6,689,604 (Issued on February 10, 2004) and US Patent No. 6,709,834 (Issued on March 23, 2004). Australian Patent No. 745040 (issued on June 20, 2002).

Gilbert, M., Young, N.M. and Wakarchuk, W.W. Fusion proteins for use in enzymatic synthesis of oligosaccharides. Australian Patent No. 754926 (Issued on March 13, 2003).

Gilbert, M. and Wakarchuk, W.W. *Campylobacter* glycosyltransferases for biosynthesis of gangliosides and ganglioside mimics. U.S. Patent No. 6,503,744 (Issued on January 1, 2003) and US Patent No. 6,699,705 (Issued on March 2, 2004). Australian Patent No. 772569 (Issued on August 12, 2004).

Gilbert, M., Wakarchuk, W.W., Young, N.M., Jennings, M.P. and Moxon, E.R. Recombinant α-2,3-sialyltransferases and their uses. U.S. Patent No. 6,210,933 (issued on April 3, 2001) and U.S. Patent No. 6,096,529 (issued on August 1, 2000). European Patent No. 0906432 (issued on June 9, 2004).

Recent publications:

Karlyshev, A.V., Champion, O.L., Churcher, C., Brisson, J.-R., Jarrell, H.C., **Gilbert, M.**, Brochu, D., St. Michael, F., Li, J., Wakarchuk, W.W., Goodhead, I., Sanders, M., Stevens, K., White, B., Parkhill, J., Wren, B.W. and Szymanski C.M. 2005. Analysis of *Campylobacter jejuni* capsular loci reveals multiple mechanisms for the generation of structural diversity and the ability to form complex heptoses. *Mol. Microbiol.* **55**:90-103.

Godschalk, P.C.R., Heikema, A.P., **Gilbert, M.**, Komagamine, T., Ang, C.W., Glerum, J., Brochu, D., Li, J., Yuki, N., Jacobs, B.C., van Belkum, A. and Endtz, H.P. 2004. The crucial role of *Campylobacter jejuni* genes in anti-ganglioside antibody induction in Guillain-Barré syndrome. *J. Clin. Invest.* **114**:1659-1665.

Zou, W., Borrelli, S., **Gilbert, M.**, Liu, T., Pon, R.A. and Jennings, H.J. 2004. Bioengineering of surface GD3 ganglioside for immunotargeting human melanoma cells. *J. Biol.Chem.* **279**: 25390-25399.

Chiu, C.P.C., Watt, A.G., Lairson, L.L., **Gilbert, M.**, Lim, D., Wakarchuk, W.W., Withers, S.G. and Strynadka, N.C.J. 2004. Structural analysis of the sialyltransferase Cst-II from *Campylobacter jejuni* in complex with a substrate analog. *Nature Struc. Mol. Biol.* **11**:163-170.

Gilbert, M., Godschalk, P.C.R., Karwaski, M.-F., Ang, C.W., van Belkum, A., Li, J., Wakarchuk, W.W. and Endtz, H.P. 2004. Evidence for the acquisition of the lipooligosaccharide biosynthesis locus in *Campylobacter jejuni* GB11, a strain isolated from a Guillain-Barré syndrome patient, by horizontal exchange. *Infec. Immun.* **72**:1162-1165.

Szymanski, C.M., St.Michael, F., Jarrell, H.C., Li, J., **Gilbert, M.**, Larocque, S., Vinogradov, E. and Brisson, J.-R. 2003. Detection of conserved *N*-linked glycans and phase-variable lipooligosaccharides and capsules from *Campylobacter* cells by mass spectrometry and high resolution magic angle spinning NMR spectroscopy. *J. Biol. Chem.* **278**:24509-24520..

Antoine, T., Priem, B., Heyraud, A., Greffe, L., **Gilbert, M.**, Wakarchuk, W.W., Lam, J.S. and Samain, E. 2003. Large-scale *in vivo* synthesis of the carbohydrate moieties of gangliosides GM1 and GM2 by metabolically engineered *Escherichia coli*. *ChemBioChem* **4**:406-412.

Yan, F., Mehta, S., Eichler, E., Wakarchuk, W.W., **Gilbert, M.**, Schur, M.J. and Whitfield, D.M. 2003. Simplifying oligosaccharide synthesis: efficient synthesis of lactosamine and sialylated lactosamine oligosaccharide donors. *J. Org. Chem.* **68**:2426-2431.

Karwaski, M.-F., Wakarchuk, W.W. and **Gilbert, M.** 2002. High-level expression of recombinant *Neisseria* CMP-sialic acid synthetase in *Escherichia coli*. *Prot. Expr. Purif.* **25**:237-240.

Priem, B., **Gilbert, M.**, Wakarchuk, W.W., Heyraud, A. and Samain, E. 2002. A new fermentation process allows large-scale production of human milk oligosaccharides by metabolically engineered bacteria. *Glycobiology* **12**:235-240.

Gilbert, M., Karwaski, M.-F., Bernatchez, S., Young, N.M., Taboada, E., Michniewicz, J., Cunningham, A.-M. and Wakarchuk, W.W. 2002. The genetic bases for the variation in the lipo-oligosaccharide of the mucosal pathogen, *Campylobacter jejuni*: biosynthesis of sialylated ganglioside mimics in the core oligosaccharide. *J. Biol. Chem.* **277**:327-337.

Yan, F., **Gilbert, M.**, Wakarchuk, W.W., Brisson, J.-R. and Whitfield, D.M. 2001. Chemoenzymatic iterative synthesis of difficult linkages of oligosaccharides on soluble polymeric supports. *Organic Lett.* **3**:3265-3268.

van Belkum, A., van den Braak, N., Godschalk, P., Ang, W., Jacobs, B., **Gilbert, M.**, Wakarchuk, W., Verbrugh, H. and Endtz, H. 2001. A *Campylobacter jejuni* gene associated with immune-mediated neuropathy. *Nature Med.* **7**:752-753.

Wakarchuk, W.W., Watson, D., St.Michael, F., Li, J., Wu, Y., Brisson, J.-R., Young, N.M, and **Gilbert, M.** 2001. Dependence of the bi-functional nature of a sialyltransferase from *Neisseria meningitidis* on a single amino acid substitution. *J. Biol. Chem.* **276**:12785-12790.

Mosimann, S.C., **Gilbert, M.**, Dombrowski, D., To, R., Wakarchuk, W. and Strynadka, N.C.J. 2001. Structure of a sialic acid-activating synthetase, CMP-acylneuraminate synthetase in the presence and absence of CDP. *J. Biol. Chem.* **276**:8190-8196.

Hood, D.W., Cox, A.D., **Gilbert, M.**, Makepeace, K., Walsh, S., Deadman, M.E., Cody, A., Martin, A., Mansson, M., Schweda, E.K.H., Brisson, J.-R., Richards, J.C., Moxon, E.R. and Wakarchuk, W.W. 2001. Identification of a lipopolysaccharide α-2,3-sialyltransferase from *Haemophilus influenzae*. *Mol. Microbiol.* **39**:341-350.

Linton, D., **Gilbert, M.**, Hitchen, P.G., Dell, A., Morris, H.R., Wakarchuk, W.W., Gregson, N.A. and Wren, B.W. 2000. Phase variation of a β-1,3 galactosyltransferase involved in generation of the ganglioside GM_1-like lipo-oligosaccharide of *Campylobacter jejuni*. *Mol. Microbiol.* **37**:501-514.

Mehta, S., **Gilbert, M.**, Wakarchuk, W.W. and Whitfield, D.M. 2000. Ready access to sialylated oligosaccharide donors. *Organic Lett.* **2**:751-753.

Gilbert, M., Brisson, J.-R., Karwaski, M.-F., Michniewicz, J., Cunningham, A.-M., Wu, Y., Young, N.M. and Wakarchuk, W.W. 2000. Biosynthesis of ganglioside mimics in *Campylobacter jejuni* OH4384: identification of the glycosyltransferase genes, enzymatic synthesis of model compounds and characterization of nanomole amounts by 600-MHz ^{1}H and ^{13}C NMR analysis. *J. Biol. Chem.* **275**:3896-3906.